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MAVS Is Essential for Primary CD4$^+$ T Cell Immunity but Not for Recall T Cell Responses following an Attenuated West Nile Virus Infection

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ABSTRACT The use of pathogen recognition receptor (PRR) agonists and the molecular mechanisms involved have been the major focus of research in individual vaccine development. West Nile virus (WNV) nonstructural (NS) 4B-P38G mutant has several features for an ideal vaccine candidate, including significantly reduced neuroinvasiveness, induction of strong adaptive immunity, and protection of mice from wild-type (WT) WNV infection. Here, we determined the role of mitochondrial antiviral signaling protein (MAVS), the adaptor protein for RIG-I-like receptor in regulating host immunity against the NS4B-P38G vaccine. We found that *Mavs*$^{-/-}$ mice were more susceptible to NS4B-P38G priming than WT mice. *Mavs*$^{-/-}$ mice had a transiently reduced production of antiviral cytokines and an impaired CD4$^+$ T cell response in peripheral organs. However, antibody and CD8$^+$ T cell responses were minimally affected. NS4B-P38G induced lower type I interferon (IFN), IFN-stimulating gene, and proinflammatory cytokine responses in *Mavs*$^{-/-}$ dendritic cells and subsequently compromised the antigen-presenting capacity for CD4$^+$ T cells. Interestingly, *Mavs*$^{-/-}$ mice surviving NS4B-P38G priming were all protected from a lethal WT WNV challenge. NS4B-P38G-primed *Mavs*$^{-/-}$ mice exhibited equivalent levels of protective CD4$^+$ T cell recall response, a modestly reduced WNV-specific IgM production, but more robust CD8$^+$ T cell recall response. Taken together, our results suggest that MAVS is essential for boosting optimal primary CD4$^+$ T cell responses upon NS4B-P38G vaccination and yet is dispensable for host protection and recall T cell responses during secondary WT WNV infection.

IMPORTANCE The production of innate cytokines induced by the recognition of pathogen recognition receptors (PRRs) via their cognate ligands are critical for enhancing antigen-presenting cell functions and influencing T cell responses during microbial infection. The use of PRR agonists and the underlying molecular mechanisms have been the major focus in individual vaccine development. Here, we determined the role of mitochondrial antiviral-signaling protein (MAVS), the adaptor protein for RIG-I like receptor in regulating host immunity against the live attenuated West Nile virus (WNV) vaccine strain, the nonstructural (NS) 4B-P38G mutant. We found that MAVS is important for boosting optimal primary CD4$^+$ T cell response during NS4B-P38G vaccination. However, MAVS is dispensable for memory T cell de-

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velopment and host protection during secondary wild-type WNV infection. Overall, these results may be utilized as a paradigm to aid in the rational development of other efficacious live attenuated flavivirus vaccines.

KEYWORDS MAVS, T cell, West Nile virus, adaptive immunity, T cells, vaccine

The production of innate cytokines induced by the recognition of pathogen recognition receptors (PRRs) via their cognate ligands is critical for enhancing antigen-presenting cell (APC) functions and influencing T cell responses during microbial infection (1–4). Thus, the involvement of PRR agonists and the molecular mechanisms involved have been the major focus of research in individual vaccine development.

Flaviviruses, a group of single-stranded, positive-sense RNA viruses, contain several pathogens of public health importance, including West Nile virus (WNV) and Zika virus (ZIKV), for which we have no licensed vaccines. WNV has caused annual outbreaks of acute viral encephalitis and neurological sequelae in North America since 1999. The genome of WNV is about 11,000 nucleotides in length, which is translated and processed into 10 proteins: three structural proteins (envelope [E], membrane, and nucleocapsid) and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (5). The NS4B protein of flaviviruses is linked to evasion of host immune responses and viral replication (6–8). The NS4B P38 residue is conserved among most mosquito- and tick-borne flaviviruses. An attenuating P38G substitution in the NS4B protein was identified previously by site-directed mutagenesis of a WNV New York 1999 strain (NY99) infectious clone (9). Animal studies suggested that the WNV NS4B-P38G mutant has multiple features of a vaccine candidate, including reduced neuroinvasiveness, induction of greater adaptive immune responses than wild-type (WT) WNV, and protection of mice from subsequent lethal WT WNV infection (10). Nevertheless, immune factors that contribute to development of strong adaptive immunity upon WNV NS4B-P38G vaccination are not well described.

WNV infection activates several PRRs, including Toll-like receptors and RIG-I-like receptors, which signal through adaptor molecules, such as TIR-domain-containing adapter-inducing beta interferon (TRIF), myeloid differentiation factor 88 (MyD88), and mitochondrial antiviral-signaling (MAVS; also known as IPS-1, VISA, or CARDIF). Signaling through these adaptors amplifies innate immune responses, culminating in the synthesis of antiviral cytokines, including type I interferons (IFNs) and proinflammatory cytokines (11–14). We have previously shown that MyD88 is required for induction of proinflammatory cytokines, but not type I IFNs during NS4B-P38G infection in myeloid dendritic cells (DCs). MyD88 deficiency also results in reduced primary and secondary T cell responses (15). Here, we investigated the role of MAVS-dependent signaling pathways in induction of innate and adaptive immune responses and host protection by the NS4B-P38G vaccine. Our results suggest that MAVS is essential for primary $CD4^+$ T cell responses but dispensable for host protection and recall T cell responses during secondary WNV infection.

RESULTS

MAVS is required for host survival after high-dose WNV NS4B-P38G priming. We previously reported that WT B6 mice were highly resistant to WNV NS4B-P38G infection. However, about 60% of NS4B-P38G-infected *Myd88*$^{-/-}$ mice survived (15). These results indicate that both MyD88-dependent and -independent immune signaling pathways contribute to host defense against NS4B-P38G infection. MAVS, the adaptor protein of RIG-I receptor is known to be important for host protection against the pathogenic WT WNV (16). Here, we investigated the role of MAVS in regulating host immunity against the NS4B-P38G vaccine strain. Initially, we infected *Mavs*$^{-/-}$ mice and WT controls with a low dose (50 PFU) or a high dose (500 PFU) of WNV NS4B-P38G. All *Mavs*$^{-/-}$ mice succumbed to 500 PFU of WNV NS4B-P38G infection in less than 2 weeks. However, 50% of *Mavs*$^{-/-}$ mice survived 50 PFU of NS4B P38G infection (Fig. 1A). All WT control mice infected with either the low or the high dose of WNV

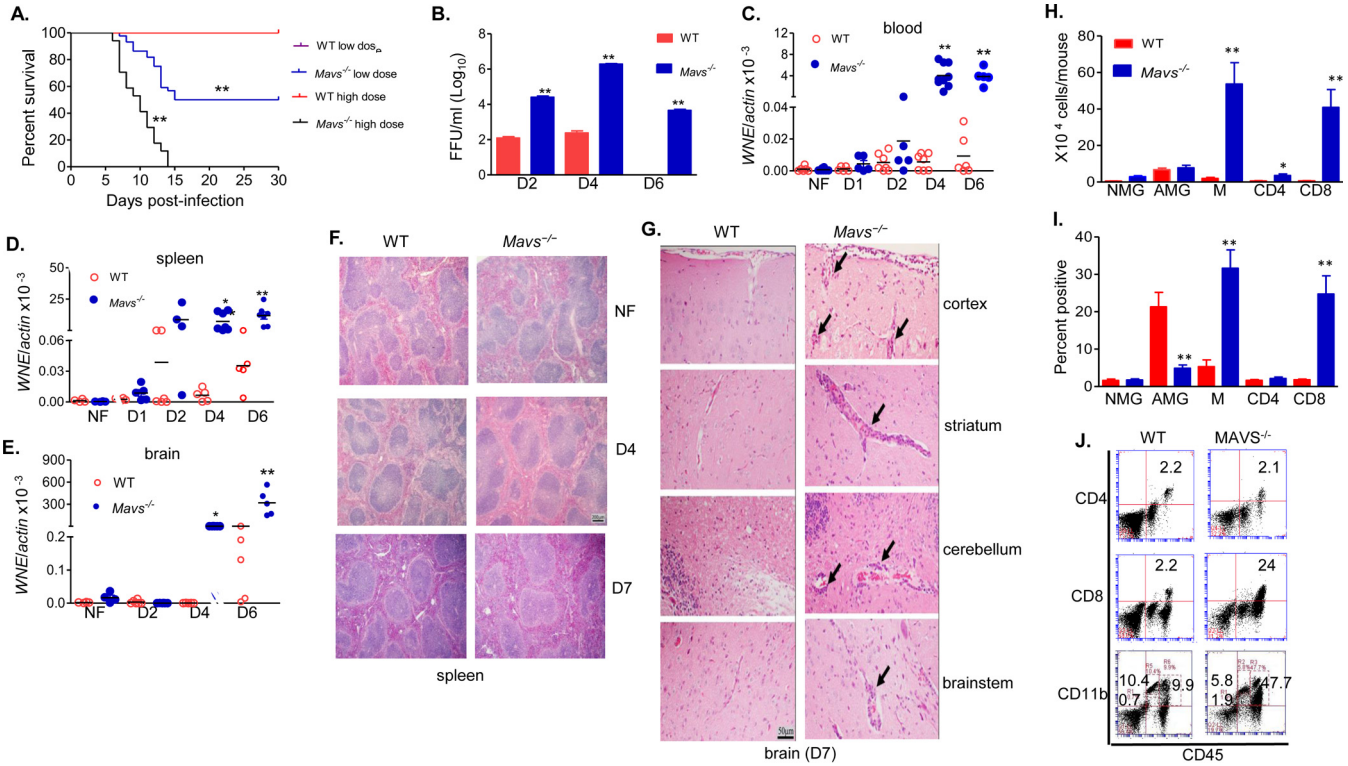

FIG 1 *Mavs*$^{-/-}$ mice are susceptible to primary WNV NS4B-P38G mutant infection. (A) Survival of WT (n = 20 or 17) and *Mavs*$^{-/-}$ (n = 44 or 17) mice after i.p. injection with a low or high dose of WNV NS4B-P38G mutant. (B and C) Viral load in blood was determined by using a FFA (B) or a Q-PCR assay (C). (D and E) Viral load in spleen (D) and brain (E) as determined by Q-PCR assay. Data are presented as means ± the SEM (n = 4 to 8). (F and G) Representative images (20×) shown are H&E-stained spleens (F) or brains (G) from NS4B-P38G-infected mice on day 4 (D4) and day 7 (D7) and/or noninfected (NF) WT and *Mavs*$^{-/-}$ mice. Arrows were pointed to mononuclear infiltrates. (H to J) Brain leukocyte infiltration following WNV NS4B P38G mutant infection. The numbers of brain leukocytes (H) or the percent positive brain leukocytes (I), including naive microglia cells (NMG), activated microglial cells (AMG), macrophages (M), and CD4$^+$ and CD8$^+$ T cells on day 7 p.i. were analyzed by flow cytometry. Data are presented as means ± the SEM (n = 8 to 10 pooled from two experiments). *, $P < 0.05$; **, $P < 0.01$ (compared to WT mice). (J) Representative images from two similar experiments are shown.

NS4B-P38G survived for at least 30 days without showing any clinical signs. Next, we measured viral loads in organs of mice infected with 500 PFU of WNV NS4B-P38G. Viremia was significantly higher in *Mavs*$^{-/-}$ mice compared to WT mice on days 2, 4, and 6 as determined by focus-forming assay (FFA) and on days 4 and 6 as determined by quantitative PCR (Q-PCR) assay (Fig. 1B and C). Viral loads in the spleen and brain were also significantly enhanced in *Mavs*$^{-/-}$ mice compared to WT mice on days 4 and 6 (Fig. 1D and E). Spleens and brains were next collected from naive and infected mice on days 4 and/or 7 for pathological examination (Fig. 1F and G). Tissue necrosis was not observed in any section of the spleen in WNV NS4B-P38G-infected WT or *Mavs*$^{-/-}$ mice. Although there was apparent germinal-center proliferation in both groups compared to noninfected mice, no differences were observed between the two groups. In the central nervous system (CNS), we noted the presence of perivascular infiltrates with mononuclear cells in the cerebral cortices and meninges, striata, cerebellar deep nuclei, and brainstems of *Mavs*$^{-/-}$ mice on day 7 postinfection (p.i.). In comparison, no obvious infiltrates were observed in WT mice at any of these sites (Fig. 1G). Furthermore, we isolated brain leukocytes and examined their phenotype. On day 7, flow cytometry analysis indicated that the number of infiltrating macrophages (CD11b^{hi} CD45^{hi}), CD4$^+$, and CD8$^+$ T cells in the brains of *Mavs*$^{-/-}$ mice increased by 5- to 65-fold compared to those of WT control mice (Fig. 1H to J). Overall, these results suggest that MAVS is essential for host protection during NS4B-P38G priming.

MAVS deficiency contributes to transiently reduced antiviral innate immune responses in peripheral tissues of NS4B-P38G-vaccinated mice. MAVS is essential for the induction of type I IFN and other innate antiviral responses during WT WNV infection (14). Type I IFNs, including both IFN-α and IFN-β, participate in the direct

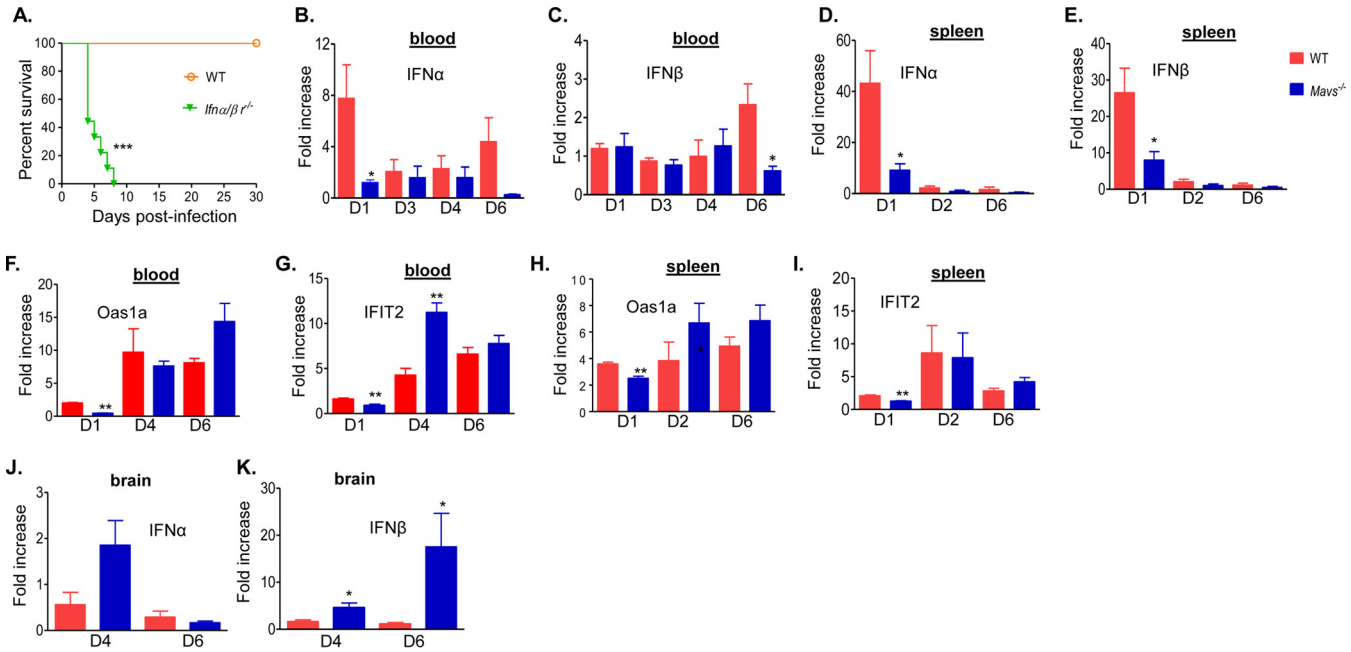

FIG 2 Type I IFN response after WNV NS4B-P38G mutant infection. (A) Survival of WT 129Sv/Ev (WT; $n = 6$) and IFN-α/βR$^{-/-}$ ($n = 9$) mice after an i.p. injection with 500 PFU of WNV NS4B-P38G. (B to E) Type I IFN expression levels in the blood (B and C) and spleens (D and E) were determined by Q-PCR assay. (F to I) ISG expression levels in the blood (F and G) and spleens (H and I) as determined by Q-PCR. (J and K) Type I IFN levels in brains of WNV NS4B-P38G-infected mice determined by Q-PCR. Data are presented as the fold increase compared to the mock-infected animals. The results are representative of three experiments ($n = 4$ to 8). **, $P < 0.01$; *, $P < 0.05$ (compared to the WT group).

control of WT WNV dissemination and clearance (17). Given the phenotype in *Mavs*$^{-/-}$ mice, we determined the role of type I IFN during WNV NS4B-P38G infection. IFN-α/β receptor-deficient (A129) mice and their WT littermate controls were injected intraperitoneally (i.p.) with 500 PFU of WNV NS4B-P38G. All A129 mice succumbed to infection within a week or so, whereas WT controls survived infection 100% without displaying any clinical signs (Fig. 2A). These results indicate that type I IFN signaling is essential for host protection against WNV NS4B-P38G infection. We next measured type I IFN levels in NS4B-P38G-infected WT and *Mavs*$^{-/-}$ mice. Blood IFN-α levels were reduced in *Mavs*$^{-/-}$ mice compared to WT controls on days 1 and 6 p.i. (Fig. 2B), whereas IFN-β levels were diminished in *Mavs*$^{-/-}$ mice only on day 6 (Fig. 2C). Splenic type I IFN levels also were decreased in the *Mavs*$^{-/-}$ group on day 1, but the differences between the two groups became insignificant at later time points (Fig. 2D and E). Levels of IFN-stimulating genes (ISGs), including *Oas1a* and *Ifit2*, were lower in both the blood (Fig. 2F and G) and spleens (Fig. 2H and I) of *Mavs*$^{-/-}$ mice on day 1 but increased to higher or equivalent levels at later time points. In the CNS, the IFN-α and IFN-β levels in the *Mavs*$^{-/-}$ group were either higher or equivalent compared to WT mice on days 4 and 6 p.i., indicating a MAVS-independent induction pathway (Fig. 2J and K).

We next evaluated the effect of MAVS signaling on proinflammatory cytokine and interleukin-10 (IL-10) levels, which correlate with greater viral infection and brain pathology in WT mice infected with WT-WNV (11, 18, 19). We observed lower levels of IL-6 and IL-12p40 on days 1, 2, and 3 p.i. with WNV NS4B-P38G in the blood of *Mavs*$^{-/-}$ mice; these levels became insignificant at later time points (Fig. 3A and B). The production of other inflammatory cytokines, including tumor necrosis factor alpha (TNF-α), IL-1β, and IFN-γ, was not different between these two groups (Table 1). Blood levels of IL-10 were higher in *Mavs*$^{-/-}$ mice on day 6 p.i. (Table 1). In the CNS, production of proinflammatory cytokines, (IL-1β, IL-6, IL-12, and TNF-α; Fig. 3C to F) and chemokines (CCL5, CXCL9, CXCL10, and MCP-1; Fig. 3G to J) were higher in *Mavs*$^{-/-}$ mice on day 6 p.i. Overall, the antiviral innate responses were diminished transiently in the peripheral tissues of *Mavs*$^{-/-}$ mice, but these responses were either unaffected or

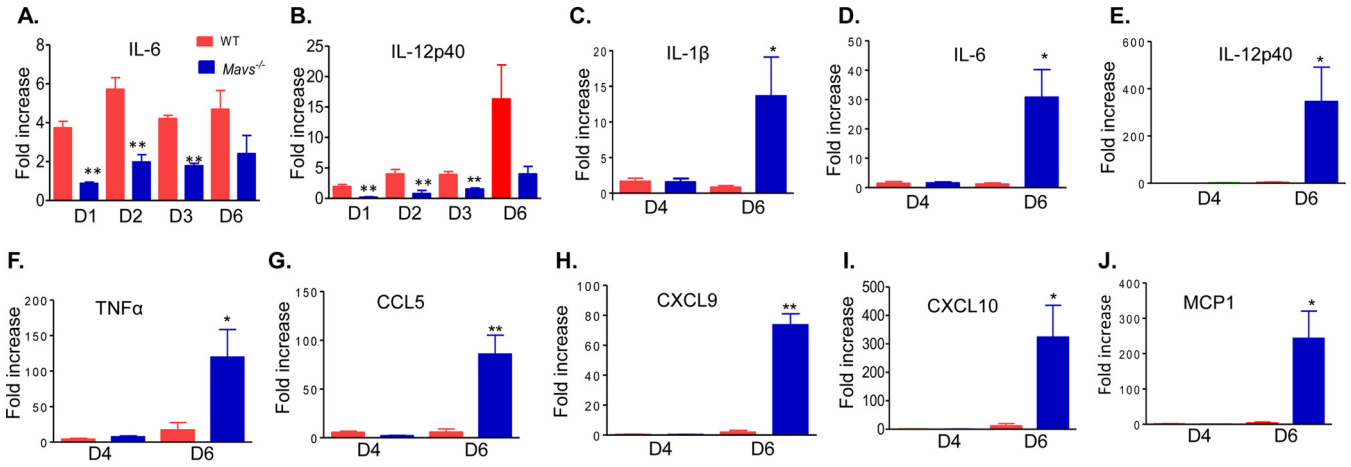

FIG 3 Inflammatory/chemotactic cytokine response after WNV NS4B-P38G mutant infection. Inflammatory cytokine and chemokine levels in blood (A and B) and in brains (C to J) at indicated time points were determined by Q-PCR assay. Data are presented as the fold increase compared to the mock-infected animals. Data are presented as means ± the SEM ($n = 4$ to 8). **, $P < 0.01$; *, $P < 0.05$ (compared to the WT group).

heightened in the CNS, indicating MAVS-independent innate immune responses in the CNS.

CD4$^+$ T cell responses, but not B cell or CD8$^+$ T cell responses, were impaired in NS4B-P38G-vaccinated *Mavs*$^{-/-}$ mice. B-cell-mediated humoral responses are critical for the host defense against disseminated infection by WT WNV (20). As shown in Fig. 4A and B, WNV-specific IgM production was only modestly diminished in the serum of *Mavs*$^{-/-}$ mice on day 4, but not at later time points. No differences were noted between the levels of serum IgG of the two groups on days 4, 6, and 7 p.i. Neither group showed neutralization titers of higher than 20 within the first week, and no differences were detected between them (data not shown). CD4$^+$ and CD8$^+$ T cells are important for host survival after WT WNV infection and contribute to long-lasting protective immunity (21, 22). Compared to WT mice, the number of IFN-γ^+ CD4$^+$ T cells and IFN-γ^+ TNF-α^+ CD4$^+$ T cells were both decreased in *Mavs*$^{-/-}$ mice on day 4 (Fig. 4C to F). No differences were observed for CD8$^+$ T cells between the two groups of mice. On day 7, CD4$^+$ T cells of NS4B-P38G-infected *Mavs*$^{-/-}$ mice produced 5-fold-less IFN-γ upon *ex vivo* stimulation with WNV-specific peptides, whereas CD8$^+$ T cells in these mice produced more IFN-γ than those of WT mice (Fig. 4G). Furthermore, CD4$^+$ T cells isolated from WNV NS4B-P38G-infected *Mavs*$^{-/-}$ mice produced less IL-2 than those of WT mice on days 4 and 7 p.i. (Fig. 4H). CD8$^+$ T cells of *Mavs*$^{-/-}$ mice also produced less IL-2 only on day 7 (Fig. 4H). In summary, CD4$^+$ T cells of *Mavs*$^{-/-}$ mice infected with WNV NS4B-P38G showed consistently lower effector function. To determine the role of primary CD4$^+$ T cells in host protection, naive *Mavs*$^{-/-}$ mice were reconstituted with CD4$^+$ T cells isolated from WT and *Mavs*$^{-/-}$ mice on day 5 postvaccination. Next, reconstituted mice were challenged with 500 PFU of WNV NS4B-P38G. Mice that received CD4$^+$ T cells from NS4B-P38G-vaccinated *Mavs*$^{-/-}$ mice all succumbed to infection, whereas about 65% of *Mavs*$^{-/-}$ mice received CD4$^+$ T cells

TABLE 1 Serum cytokine levels at days 2 and 6 postinfection[a]

	Mean cytokine level (pg/ml) ± SD			
	Day 2		Day 6	
Cytokine	WT mice	*Mavs*$^{-/-}$ mice	WT mice	*Mavs*$^{-/-}$ mice
IL-1β	264.8 ± 71.9	143.6 ± 22.2	591 ± 103	588.4 ± 41.6
TNF-α	2,232 ± 886	1,772 ± 343.5	4,230 ± 774	4,212 ± 691
IFN-γ	39.4 ± 17.9	46.6 ± 15.3	1,364 ± 272.3	1,752 ± 240
IL-10	245.7 ± 78.1	130.4 ± 21.2	140.4 ± 32.7	417.1 ± 49.5†

[a]Blood was harvested from WNV-infected mice at the indicated time points postinfection. Serum cytokine levels were measured using a Bio-Plex assay. *, $P < 0.05$; †, $P < 0.01$ (compared to WT group; $n = 4$ to 5).

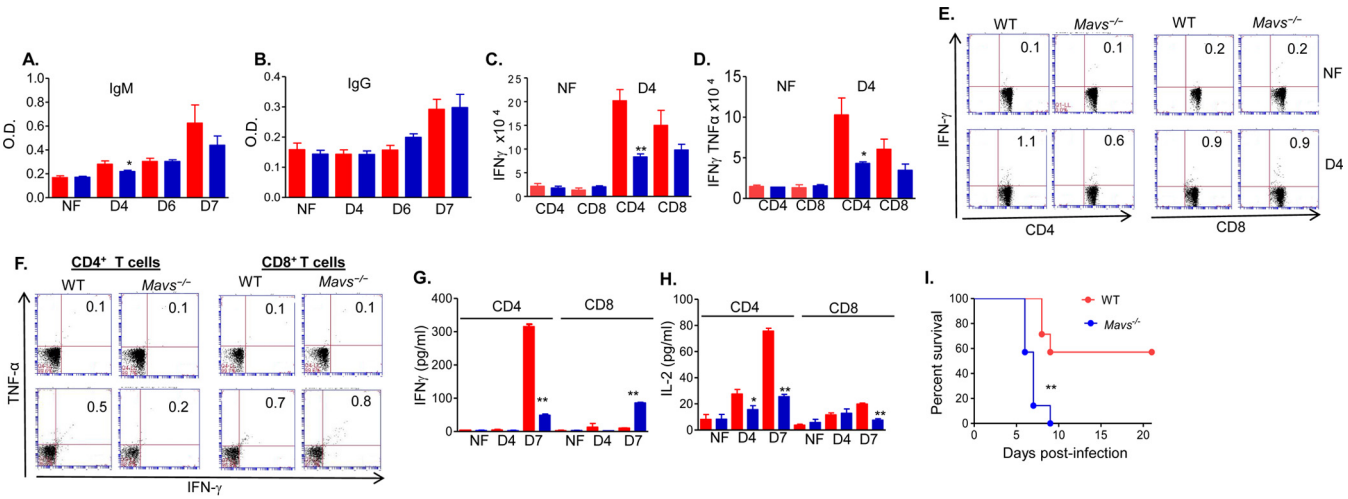

FIG 4 Antibody and T cell responses after primary infection with WNV NS4B-P38G. (A and B) Humoral responses during primary WNV NS4B-P38G infection. Sera were collected from noninfected mice (NF) or from mice infected with WNV NS4B-P38G mutant at days 4, 6, and 7 after primary infection. The development of WNV-specific IgM (A) or IgG (B) antibodies was determined by ELISA. (C to F) Splenocytes were harvested at days 0 and 4 after primary WNV NS4B-P38G infection, cultured *ex vivo* with WNV peptides for 5 h, and then stained for IFN-γ, TNF-α, and T cell markers. The total numbers of IFN-γ^+ (C) and IFN-γ^+ TNF-α^+ (D) T cell subsets per spleen are indicated. (E and F) Representative results from three similar experiments. (G and H) Splenocytes were harvested at days 0, 4, and 7 after primary WNV NS4B-P38G infection and cultured *ex vivo* with WNV-specific peptides for 3 days; IFN-γ and IL-2 production was then measured in the cell culture supernatant. **, $P < 0.01$; *, $P < 0.05$ (compared to WT mice). $n = 4$ to 5 mice/group pooled from two separate experiments. (I) Survival of naive *Mavs*$^{-/-}$ mice transferred with CD4$^+$ T cells of day 5 vaccinated WT ($n = 7$) or *Mavs*$^{-/-}$ mice ($n = 7$), followed by challenge with 500 PFU of WNV NS4B-P38G.

of NS4B-P38G-vaccinated WT mice survived infection (Fig. 4I). These results further indicate that CD4$^+$ T cells of *Mavs*$^{-/-}$ mice have impaired antiviral responses.

NS4B-P38G triggered lower type I IFN, ISG, and proinflammatory responses in *Mavs*$^{-/-}$ DCs and compromised the antigen-presenting capacity for CD4$^+$ T cells. DCs are antigen-presenting cells (APCs) that initiate T cell responses and are permissive to WT WNV infection (23). The NS4B-P38G replication was enhanced in *Mavs*$^{-/-}$ DCs (Fig. 5A and B) and remained high on day 4 p.i. compared to WT DCs, where it was cleared. Type I IFN levels were reduced on day 1 and/or day 4 in *Mavs*$^{-/-}$ DCs compared to the WT cells (Fig. 5C and D). Inflammatory cytokines, including IL-6 and IL-12, were also diminished in *Mavs*$^{-/-}$ DCs (Fig. 5E and F), although no differences were detected in the production of other inflammatory cytokines, such as TNF-α and IL-1β (data not shown). In assessing APC functions, we found that the percentage of CD80 (Fig. 5G) and major histocompatibility complex class II (MHC-II) expression (Fig. 5I) was lower for DCs isolated from NS4B-P38G-infected *Mavs*$^{-/-}$ mice on day 3 compared to WT controls. No differences were noted on CD86 (Fig. 5H) between the DCs of the two groups (data not shown). Consistent with these results, using an *in vitro* T cell priming assay, we observed that the DCs of *Mavs*$^{-/-}$ mice cocultured with OT-II T cells produced less IL-2 (Fig. 5J) and showed reduced T cell proliferation (Fig. 5K and L) compared to WT cells. Taken together, these results indicate that a deficiency of MAVS results in less activation and compromised APC functions in DCs during WNV NS4B-P38G infection.

To further understand the role of MAVS-mediated innate signaling in DC activation, we next analyzed the expression of a panel of WNV-inducible genes by Q-PCR array. As shown in Fig. 6A and B, the levels of many ISGs, including *Irf7*, *Ifi44*, *Isg15*, *Oas1a*, *Rsad2*, *Ifit1*, *Ifti2*, *Ifi27a*, *Ddx58*, *Ccl2*, and *Cxcl10*, were reduced by >1.5-fold in NS4B-P38G-infected *Mavs*$^{-/-}$ DCs compared to WT cells. In contrast, *Irf3*, *Irf5*, *Ccl5*, and *NFkb1* gene expression levels were slightly increased in NS4B-P38G-infected *Mavs*$^{-/-}$ DCs compared to WT cells. Irf3 and Irf7 are two ISGs important for IFN induction upon WT WNV infection in myeloid DCs (23, 24). Here, DCs of *Irf3*$^{-/-}$, *Irf7*$^{-/-}$, or *Irf3*$^{-/-}$ *Irf7*$^{-/-}$ mice displayed a transient reduction of type I IFN expression and enhanced viral replication on day 1. However, the viral load was reduced in all types of DCs by day 4 (Fig. 6C, D, and E). These results suggest that IRF3 and IRF7 are transiently involved in the control

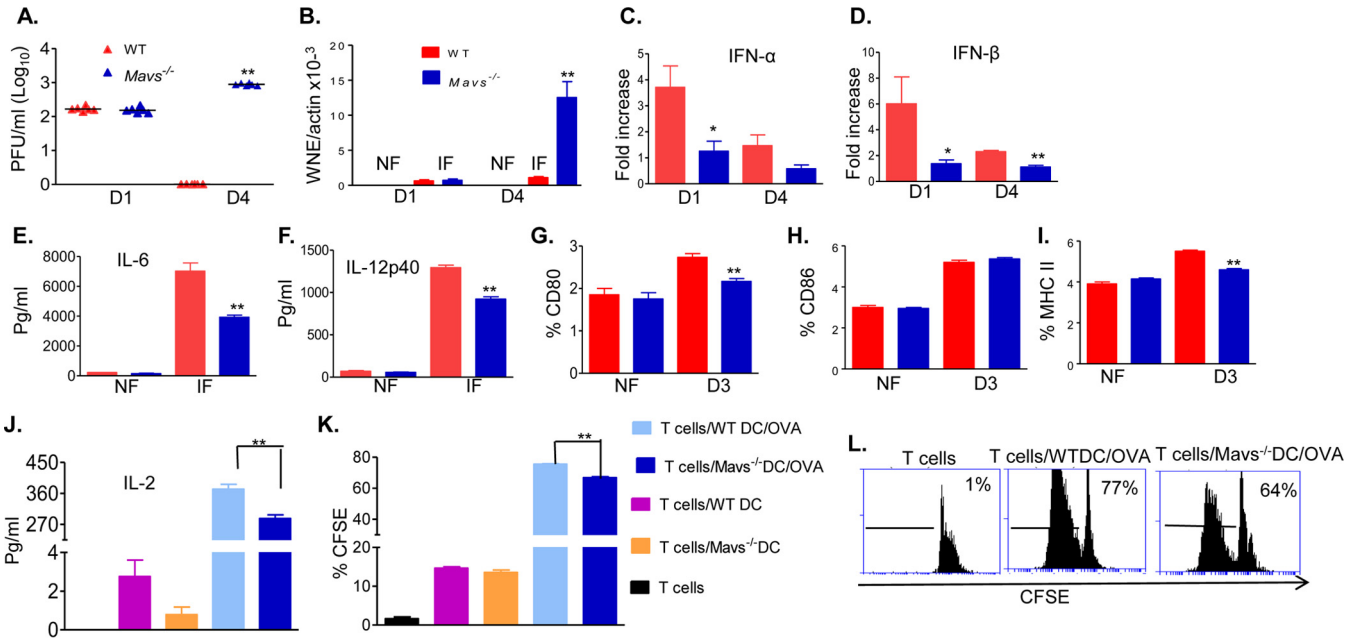

FIG 5 WNV NS4B-P38G infection in DCs. WT and *Mavs*$^{-/-}$ DCs were infected with WNV NS4B-P38G. (A and B) The viral load was measured at the indicated time points by FFA (A) and Q-PCR assay (B). (C to F) Cytokine production was assessed by Q-PCR (C and D) or Bio-Plex assays (E and F). (G to I) CD80, CD86, and MHC-II expression on DCs isolated from naive or NS4B-P38G-infected mice on day 3 p.i. (J to L) T cell priming assay. CFSE-labeled CD4$^+$ T cells from naive OT-II transgenic mice were cocultured with DCs from WNV-NS4B-P38G mutant-infected WT or *Mavs*$^{-/-}$ mice in the presence or absence of the OVA 323-339 peptide. Cells and supernatant were harvested at day 5 and analyzed for IL-2 production (J) and T cell proliferation (K and L). $n = 4$. *, $P < 0.05$; **, $P < 0.01$ (compared to the WT group).

of NS4B-P38G replication in DCs. Overall, MAVS is required for the induction of type I IFNs, the control of viral replication, and the activation of myeloid DCs upon WNV NS4B-P38G infection.

MAVS is not required for host protection and development of WNV-specific T cell recall responses upon secondary challenge. To determine the role of MAVS in long-lasting host immunity, WT and *Mavs*$^{-/-}$ mice were injected i.p. with 50 PFU of WNV NS4B-P38G mutant. On day 30 p.i., we examined CD4$^+$ and CD8$^+$ T cell effector functions in the surviving mice. Upon *ex vivo* stimulation with WNV-specific peptides, there were no differences in the number of IFN-γ^+ CD4$^+$ T cells between the two groups of mice, whereas *Mavs*$^{-/-}$ mice had 2-fold more IFN-γ^+ CD8$^+$ T cells than WT mice. Interestingly, the numbers of effector memory CD4$^+$ (CD27$^+$ CD62L$^-$) and CD8$^+$ (CD127$^+$ CD62L$^-$) T cells were enhanced in *Mavs*$^{-/-}$ mice compared to WT mice (Fig. 7A and B). Similar results were observed for splenic CD8$^+$ T cell response at 2.5 months p.i., whereas the splenic CD4$^+$ T cells of NS4B-P38G-infected *Mavs*$^{-/-}$ mice produced more IFN-γ upon *ex vivo* stimulation with WNV-specific peptides (Fig. 7C and D). No differences were noted in brain T cell responses between these two groups of mice (Fig. 7E and F). Both groups of mice had similar levels of WNV-specific antibody responses on day 30 p.i. (Fig. 7G to I). Next, surviving mice from both groups were challenged with a lethal dose of WT WNV. Remarkably, both WT and *Mavs*$^{-/-}$ mice survived infection without showing any clinical signs of disease (Fig. 8A). There were no detectable viremia in either group at day 4 after rechallenge (Fig. 8B and C). However, WNV-specific IgM production was lower in *Mavs*$^{-/-}$ mice than in WT mice (Fig. 8D). There were no differences in IgG response or neutralization titers between the two groups (Fig. 8E and F). The percentages and numbers of IFN-γ^+ CD4$^+$ T cells and IFN-γ^+ TNF-α^+ CD4$^+$ T cells also were equivalent between the two groups of mice. In contrast, there was a greater CD8$^+$ T cell response in *Mavs*$^{-/-}$ mice compared to WT mice (Fig. 8G to K). IL-2 production from CD4$^+$ or CD8$^+$ T cells of both groups of mice was similar (Fig. 8L). To determine the role of CD4$^+$ T cell recall response in host protection during secondary infection, naive *Mavs*$^{-/-}$ mice were adoptively transferred with CD4$^+$ T cells

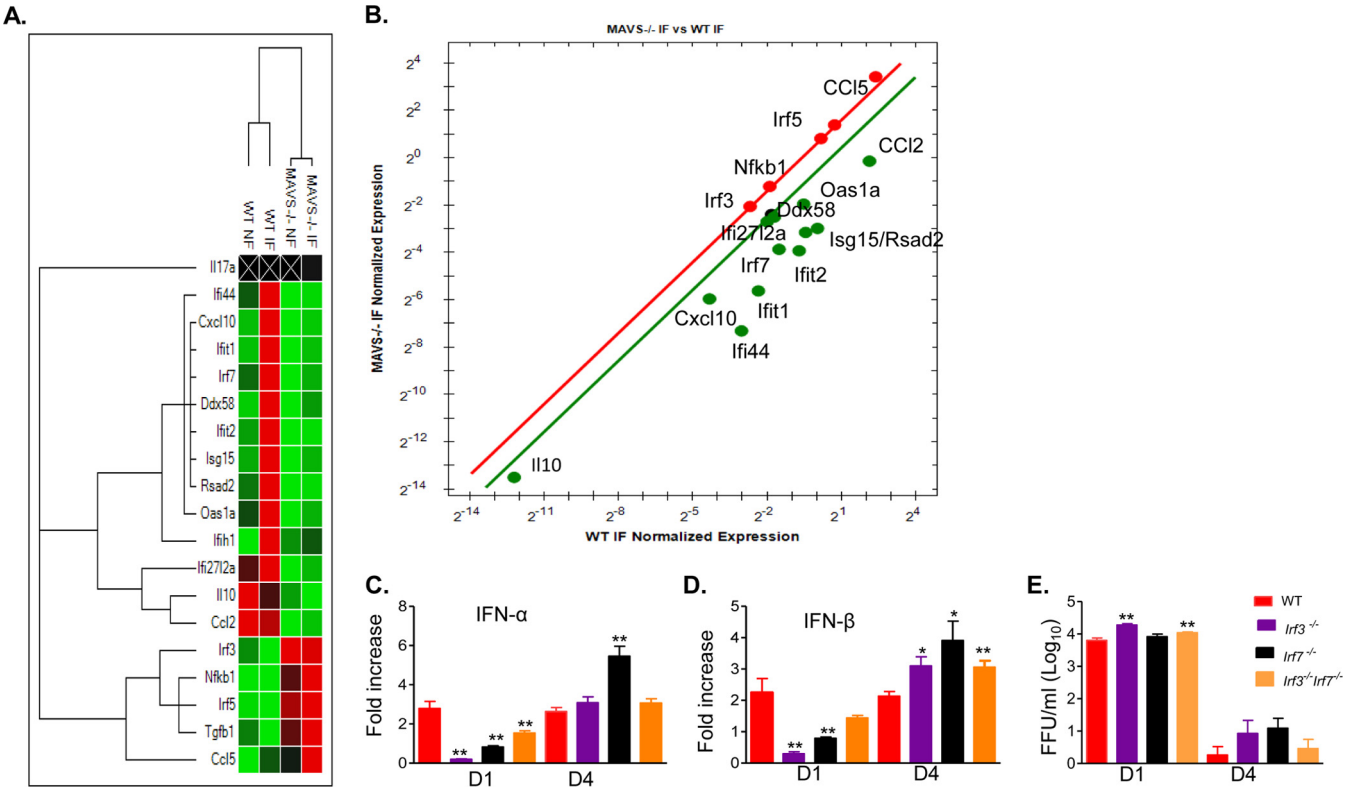

FIG 6 ISGs induction following WNV NS4B-P38G infection in BM-DCs. (A and B) PrimePCR array. (A) Clustergram image depicting the relative expression of a sample. Upregulation (higher expression) is indicated by a red square, downregulation (lower expression) is indicated by a green square, and no regulation is indicated by a black square. On the outer edges of the data plot is a dendrogram, which indicates the clustering hierarchy. (B) Scatter plot showing the normalized expression of targets for NS4B-P38G-infected WT versus *Mavs*$^{-/-}$ DCs. The plot image shows the changes in target expression based on the threshold set of 1.5. Upregulation is indicated by red circles, downregulation is indicated by green circles, and no change is indicated by black circles. (C to E) WNV NS4B-P38G infection in BM-DCs of WT, *Irf3*$^{-/-}$, *Irf7*$^{-/-}$, and *Irf3*$^{-/-}$ *Irf7*$^{-/-}$ mice. (C and D) Cytokine expression was determined by Q-PCR. (E) Viral load was measured at the indicated time points by FFA. The results are representative of two experiments ($n = 5$ to 6). **, $P < 0.01$; *, $P < 0.05$ (compared to the WT group).

isolated from WT or *Mavs*$^{-/-}$ mice on day 30 p.i., followed by infection with 500 PFU of WNV NS4B-P38G. Similar survival rates were observed between mice transferred with CD4$^+$ T cells from NS4B-P38G-infected WT and *Mavs*$^{-/-}$ mice (Fig. 8M). Overall, these results suggest that MAVS is not required for host protection during secondary challenge with WT WNV after priming with the NS4B-P38G mutant. In addition, MAVS deficiency either has no effect or even promotes T cell recall responses during secondary challenge.

DISCUSSION

Upon viral infection, activation of multiple PRRs leads to the production of type I IFNs, proinflammatory cytokines, and chemokines. The innate immune products of PRR signaling are directly involved in viral clearance. More evidence suggests that they also contribute to activation of adaptive immunity via promoting APC maturation and/or directly regulating the functions of adaptive immune cells (1–4). In this study, our results suggest that MAVS is required for host protection during priming with NS4B-P38G but is not essential for protecting host from secondary infection with a lethal dose of WT WNV. A deficiency of MAVS in NS4B-P38G-vaccinated mice resulted in a transiently reduced production of antiviral cytokines and impaired primary CD4$^+$ T cell activities in peripheral organs. CD4$^+$ T cells are known to play a direct role in limiting viral replication during WT WNV infection (25). We also found that higher viral loads in the CNS led to the induction of type I IFNs, inflammatory cytokines, and chemokines in a MAVS-independent manner. Thus, the impaired CD4$^+$ T cell activities and antiviral innate responses in the periphery organs contribute to a higher viral load, more virus

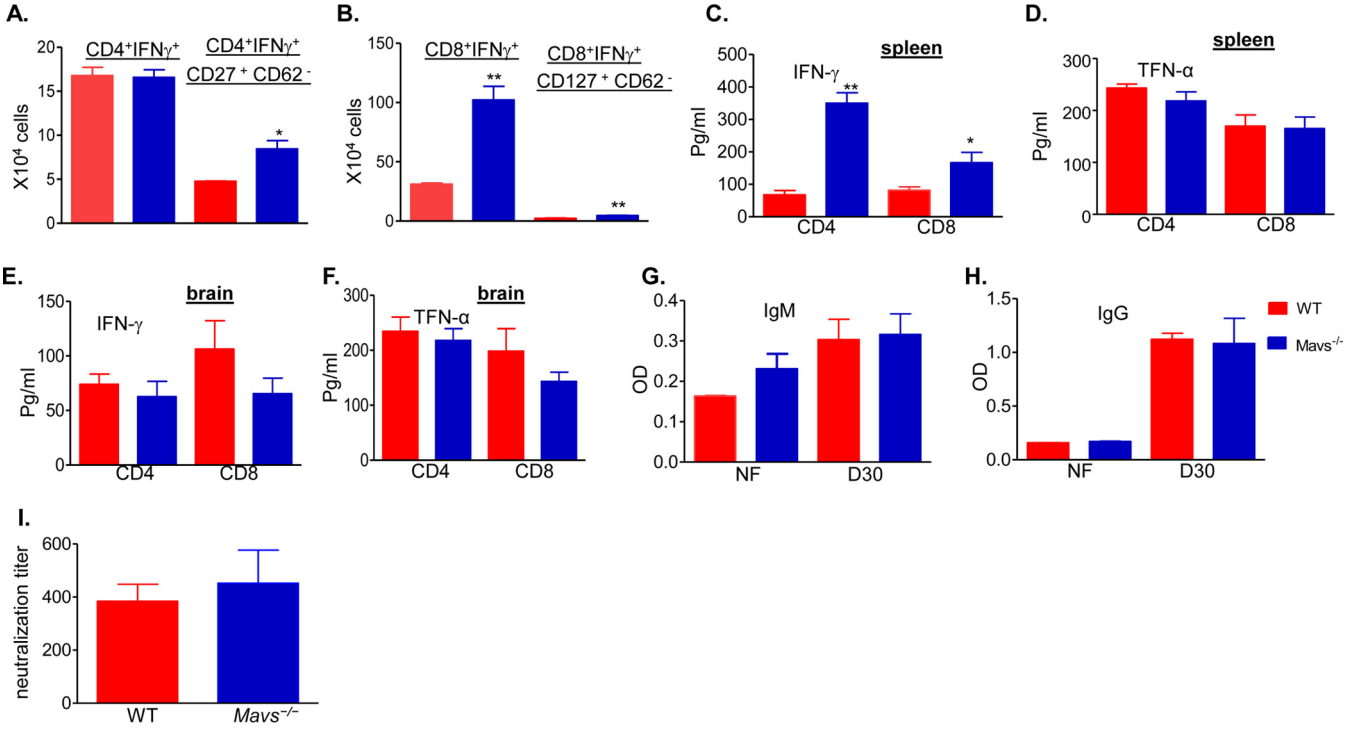

FIG 7 Antibody and T cell responses in WNV NS4B-P38G-infected mice before rechallenge. (A and B) Splenocytes were harvested at day 30 after NS4B-P38G primingm cultured *ex vivo* with WNV peptides for 5 h, and stained for IFN-γ, T cell markers, and CD62L, CD27, or CD127. The total numbers of IFN-γ^+, IFN-γ^+ CD27$^+$ CD62L$^-$ CD4$^+$, or CD8$^+$ T cell subsets per spleen are shown. (C to F) Splenocytes (C and D) or brain leukocytes (E and F) isolated from both groups of mice at 2.5 months p.i. and were cultured *ex vivo* with WNV peptides for 3 days. IFN-γ and TNF-γ production was measured in the cell culture supernatant. (G to I) Humoral response. Sera were collected from noninfected (NF) or mice infected with WNV NS4B-P38G mutant at day 30 p.i. WNV-specific IgM (G) or IgG (H) antibodies were determined by ELISA or neutralization assay (I).

dissemination, and greater inflammatory cell infiltration into the CNS during priming with NS4B-P38G. However, NS4B-P38G-vaccinated *Mavs*$^{-/-}$ mice exhibited equivalent levels of protective CD4$^+$ T cell recall response during secondary WT WNV infection. Type I IFNs and proinflammatory cytokines are known to promote DC maturation (26, 27). Here, NS4B-P38G induced lower type I IFNs, IL-6 and IL-12 production in *Mavs*$^{-/-}$ DCs compared to WT DCs, thereby compromising their antigen-presenting capacity. Furthermore, IL-1β, IL-6, and TNF-α can act directly on naive T cells to provide a third signal, to synergize with signals from the TCR and costimulatory receptors, and to regulate the expansion and survival of CD4$^+$ memory T cells (28). We found that MAVS deficiency resulted in a transient reduction of blood IL-6 and IL-12 levels during early stage of infection with NS4B-P38G but recovered to normal levels at a late stage of infection. The production of other inflammatory cytokines, such as IL-1β and TNF-α, was similar between the two groups. Thus, these results support the observation that CD4$^+$ T cell recall responses were not affected in the absence of MAVS.

Current research on the role of MAVS in adaptive immunity has been focused mainly on humoral and primary CD8$^+$ T cell responses. For example, it was reported that MAVS deficiency dramatically inhibited T-independent antibody responses (29). However, we only observed a modest decrease on WNV-specific IgM responses but no changes in neutralization activity in *Mavs*$^{-/-}$ mice during NS4B-P38G priming and secondary WT WNV infection. There are contradictory reports about the role of MAVS signaling in regulating CD8$^+$ T cell response. One early report showed that during infection with hepatitis C virus, another member of the *Flaviviridae* family, despite the NS3/4A-mediated cleavage of mouse MAVS, infected murine hepatocytes were eliminated by hepatic CD8$^+$ T cells, indicating unaffected effector functions (30). Two other groups also demonstrated that MAVS is not required for cytotoxic activities or recruitment of CD8$^+$ T cells into the lungs and airways during respiratory syncytial virus infection (31,

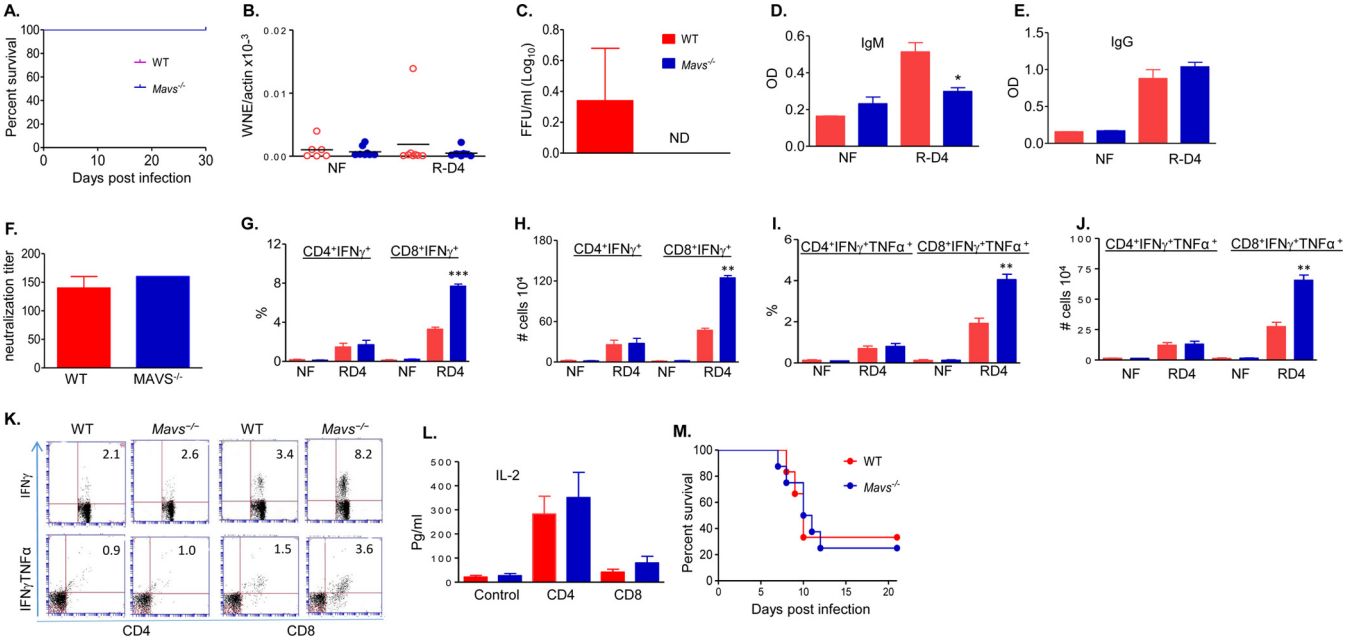

FIG 8 Antibody and T cell responses in WNV NS4B-P38G-infected mice during secondary challenge. (A) Survival rate. WT ($n = 15$) and *Mavs*$^{-/-}$ ($n = 16$) mice that survived primary WNV NS4B-P38G mutant infection were rechallenged with lethal WT WNV at day 30. (B and C) Viremia on day 4 after rechallenge (R-D4) was determined by using a Q-PCR assay (B) or an FFA (C) ($n = 6$ to 8). (D to F) Humoral response during secondary WNV infection. Sera were collected from noninfected mice (NF) or mice infected with WNV NS4B-P38G mutant at day 4 after rechallenge (R-D4) with WT WNV. WNV-specific IgM (D) or IgG (E) antibodies were determined by ELISA or neutralization assay (F). (G to K) Splenocytes were harvested from noninfected (NF) or mice at day 4 after rechallenge with WT WNV (R-D4), cultured *ex vivo* with WNV peptides for 5 h, and then stained for IFN-γ, TNF-α, and T cell markers. The percentages and total numbers of IFN-γ$^+$ (G and H) and IFN-γ$^+$ TNF-α$^+$ (I and J) T cell subsets per spleen are shown. (K) Representative image of three similar experiments is shown. (L) Splenocytes were cultured *ex vivo* with WNV peptides for 3 days, and IL-2 production was measured in the cell culture supernatant. (M) Survival of naive *Mavs*$^{-/-}$ mice transferred with CD4$^+$ T cells of day 30 NS4B-P38G-infected WT ($n = 6$) or *Mavs*$^{-/-}$ ($n = 8$) mice, followed by challenge with 500 PFU of WNV NS4B-P38G.

32). Nevertheless, activation of MAVS signaling induces the production of type I IFNs, which are the main candidate signal 3 cytokines for CD8$^+$ T cells in response to many intracellular pathogens (1–3, 33, 34). Accordingly, during WT WNV infection, blockade of type I IFN signaling starting on day 4 was shown to induce dysfunctional CD8$^+$ T cell response with exhaustion phenotypes (35). In this study, we found that type I IFN levels of NS4B-P38G-vaccinated *Mavs*$^{-/-}$ mice were decreased in serum but maintained at normal levels in the spleen after day 1. This partial reduction of type I IFN did not seem to affect CD8$^+$ T cell activation in *Mavs*$^{-/-}$ mice. Compared to WT mice, primary CD8$^+$ T cells showed normal effector functions in periphery organs and maintained the capacity to migrate into the CNS within the first week postinfection. Furthermore, *Mavs*$^{-/-}$ mice exhibited stronger CD8$^+$ memory T cell activity on day 30 postpriming and had more robust recall response upon rechallenge with WT WNV infection. It was reported that the initial viral load correlates with the magnitude of CD8$^+$ T cell responses during immunization with the yellow fever virus vaccine (YFV-17D) (36). NS4B-P38G-vaccinated *Mavs*$^{-/-}$ mice showed enhanced viral loads and delayed viral clearance in tissues, which may contribute to a higher magnitude of CD8$^+$ T cell recall responses and a mild increase on the number of CD4$^+$ effector memory T cells. In summary, our study suggests that MAVS is dispensable for the development of memory CD4$^+$ and CD8$^+$ T cells in NS4B-P38G-vaccinated mice.

Analogous to our studies with NS4B-P38G, MAVS was previously shown to be essential for host protection against the pathogenic WT WNV infection (16, 37). MAVS is important for induction of type I IFNs and control of WT WNV replication in DCs. MAVS deficiency is also associated with increased CNS inflammation. However, WT WNV and NS4B-P38G-infected *Mavs*$^{-/-}$ mice displayed differential innate and adaptive immune responses. For example, there were elevated systemic type I IFN, proinflammatory cytokine and chemokine responses, enhanced humoral responses with significantly reduced neutralization activity, and increased numbers of virus-specific CD8$^+$ T

cells in the periphery of WT WNV-infected *Mavs*$^{-/-}$ mice. Furthermore, there were equivalent number of CD4^{+} T cells in both groups of mice on day 6 post WT WNV infection. Previous studies also showed that MAVS-dependent induction of ISGs upon WT WNV infection can occur through an IRF-5-dependent, yet IRF-3- and IRF-7-independent pathway (23). Here, we noticed a mild increase on IRF5 expression in NS4B-P38G-infected *Mavs*$^{-/-}$ DCs compared to WT DCs (Fig. 6). The underlying mechanisms of MAVS-induced differential immune responses are not clearly understood. NS4B protein of flaviviruses has been associated with evasion of host innate immunity (6–8, 38, 39). In particular, the highly conserved N-terminal domain (amino acids 35 to 60) of NS4B protein bears a resemblance to an immunomodulatory tyrosine inhibitory motif found in various components of mammalian cell signaling cascades (40), which is thought to contribute to its antagonist activities for IFN and inflammatory cytokine signaling (7, 8, 38). We have previously shown that NS4B-P38G induces higher innate and adaptive T cell response than wild-type WNV (10). Thus, it is likely that the P38G substitution contributes to MAVS-induced differential innate and adaptive immune responses upon infection with WT WNV and the NS4B-P38G vaccine strain.

Live attenuated vaccines, which induce durable protective immunity, constitute an important strategy to control flavivirus infections, including yellow fever and Japanese encephalitis viruses and, more recently, dengue virus. PRR-mediated type I IFNs and proinflammatory cytokines synergize with signals from the T cell and costimulatory receptors to induce the functional maturation of T cells upon pathogen infection (1–3). The use of PRR agonists has been a major focus of research in vaccine development against these viral diseases. For example, activation of the RIG-I pathway promotes T follicular helper cell induction during influenza vaccination and protective immunity (41). Our investigation of the role of PRR-mediated signaling pathways in regulation of WNV NS4B-P38G mutant induced protective adaptive immunity may provide a model for the rational development and optimization of other live attenuated flavivirus vaccines, including ZIKV.

MATERIALS AND METHODS

Mice. Six- to ten-week-old WT C57BL/6 (B6), 129Sv/Ev, and OT-II (B6) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). *Ifnαβr*$^{-/-}$ mice in a 129Sv/Ev background or (A129), *Mavs*$^{-/-}$, *Irf3*$^{-/-}$, *Irf7*$^{-/-}$, *Irf3*$^{-/-}$ *Irf7*$^{-/-}$ mice (all in a B6 background) have been described previously (16, 23) and were bred in a pathogen-free mouse facility. This study was carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health). All animal experiments were approved by the Animal Care and Use Committee at the University of Texas Medical Branch. The WNV NS4B-P38G mutant was produced by site-directed mutagenesis and passaged twice in Vero cells (9). The parental strain WNV NY99, kindly provided by R. Tesh, was passaged once in Vero cells and twice in C6/36 cells. Mice were inoculated i.p. with 50 or 500 PFU of WNV NS4B-P38G mutant. In some experiments, mice were rechallenged with a 100% lethal dose (LD_{100}; 2,500 PFU) of WNV at day 30 after primary infection. Infected mice were monitored twice daily for signs of morbidity.

Infection of cells. Bone marrow (BM)-derived DCs were generated as described previously (23). Briefly, BM cells were cultured for 6 days in medium supplemented with granulocyte-macrophage colony-stimulating factor and IL-4 (Peprotech) to generate myeloid DCs. Cells were infected with WNV at a multiplicity of infection of 0.2, and supernatants and cells were collected at 24 and 96 h p.i. to measure cytokine production.

Focus-forming assay. Vero cell monolayers were incubated with sample dilutions first for 1 h. A semisolid overlay containing 0.8% methylcellulose (Sigma-Aldrich, St. Louis, MO), 3% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% L-glutamine (Invitrogen) was then added. At 48 h, the semisolid overlay was removed, cell monolayers were washed with phosphate-buffered saline (PBS), air dried, and fixed with acetone-methanol (1:1) solution for at least 30 min at −20°C. Cells were incubated with a rabbit WNV polyclonal antibody (kindly provided by R. Tesh), followed by goat anti-rabbit horseradish peroxidase-conjugated IgG (KPL, Gaithersburg, MD) at room temperature for 1 h, respectively. Subsequently, the cells were incubated with a peroxidase substrate (Vector Laboratories, Burlingame, CA) until color developed. The number of foci was determined manually and used to calculate viral titers expressed as FFU/ml. The limit of detection for the assay was determined to be 90 FFU/ml.

Q-PCR and PCR array. WNV-infected samples were resuspended in TRIzol (Invitrogen) for RNA extraction. Complementary DNA (cDNA) was synthesized using a qScript cDNA synthesis kit (Bio-Rad, Hercules, CA). The sequences of the primer sets for WNV envelope (*WNVE*), cytokine, and ISG cDNA and PCR conditions were as described previously (11, 23, 42, 43). The assay was performed in the CFX96 real-time PCR system (Bio-Rad). Gene expression was calculated either based on C_T values by using the formula $2^{-[CT(\text{target gene})-CT(GAPDH)]}$, as described previously (44), or the ratio of the amount of amplified gene compared with the amount of *β-actin* cDNA as the relative levels in each sample. A 25-gene

PrimePCR assay (Bio-Rad) was performed according to the manufacturer's protocol. Briefly, RNA was purified from noninfected and WNV-infected cells by using an RNeasy extraction kit (Qiagen, Valencia, CA) and quantitated by spectrometry. cDNA was synthesized by using iScript family reverse transcription kits and then loaded onto 96-well PCR array plates for amplification on the CFX96 real-time PCR system (Bio-Rad). The list of 25 genes was described previously (43). Data analysis was performed by using CFX manager software.

Brain leukocyte isolation. Brain leukocytes were isolated at day 7 or 2.5 months p.i. based on a previously described method (45). After extensive cardiac perfusion to deplete intravascular leukocytes, brains were collected and homogenized. The cell homogenates were centrifuged and resuspended in 7 ml of PBS with 2% FBS mixed with 3 ml of 90% Percoll (Sigma-Aldrich) in PBS. The cell suspension was placed under 1 ml of 70% Percoll in RPMI 1640 and centrifuged at 800 $\times$ *g* for 20 min at 22°C. Leukocytes at the interface were harvested and counted.

Cytokine Bio-Plex. Tissue culture supernatants or mouse sera were collected for analysis of cytokine production using a Th17 6-Plex or 23-Plex Bio-Plex Pro mouse cytokine assay (Bio-Rad).

Enzyme-linked immunosorbent assay (ELISA). Microtiter plates were coated with recombinant WNV E protein (45) overnight at 4°C at 50 ng/well. Sera were diluted 1/30 in PBS with 2% bovine serum albumin, followed by incubation for 1 h at room temperature. Alkaline phosphatase-conjugated goat anti-mouse IgG or IgM (Sigma-Aldrich) at a dilution of 1/1,000 in PBS-Tween was then added for 1 h. Color was developed with *p*-nitrophenyl phosphate (Sigma-Aldrich), and the intensity was determined at an absorbance of 405 nm using a spectrophotometer.

Plaque reduction neutralization tests (PRNTs). Neutralizing antibody titers were determined using a 50% neutralization cutoff ($PRNT_{50}$). Serum was diluted 1:5 in PBS and heat inactivated at 56°C for 30 min before preparing 2-fold dilutions in 100 μl of maintenance medium. The parental virus was diluted to 200 PFU/100 μl, and a 100-μl portion of virus was added to 100 μl of serially diluted serum, mixed, and placed at 4°C overnight. Then, 100 μl of the serum-virus mixture was added to Vero cells in 6-well plates. The plaques were counted, and the $PRNT_{50}$ was determined.

Histology. Mice were perfused extensively with PBS. Brains and spleens were removed and placed in 4% paraformaldehyde (PFA) for 3 days at 4°C, followed by 70% ethanol for 24 h before they were embedded in optimal cutting temperature compound. Samples were prepared for hematoxylin and eosin (H&E) staining at the Histopathology Laboratory Core at Baylor College of Medicine (Houston, TX).

Flow cytometry. Splenocytes were stained with antibodies for CD11c, CD80, CD86, CD3, CD4, or CD8 (e-Biosciences, San Diego, CA). Isolated brain leukocytes were stained with antibodies for cell surface markers, including CD3, CD4, CD8, CD11b, and CD45 (BD Biosciences). After staining, the cells were fixed with 1% paraformaldehyde in PBS and examined using a C6 flow cytometer (BD Biosciences). Dead cells were excluded on the basis of forward and side light scatter. Data were analyzed with a CFlow Plus flow cytometer (BD Biosciences).

Intracellular cytokine staining. Splenocytes were stimulated with WNV-specific NS3 and E peptides (RRWCFDGPRTNTILE and PVGRLVTVNPFVSVA, respectively [25]) for $CD4^+$ T cells or with WNV-specific NS4B and E peptides (SSVWNATTA and IALTFLAV, respectively [46]) for $CD8^+$ T cells for 5 h at 37°C in the presence of Golgi-plug (BD Biosciences). Cells were stained with antibodies for CD3, CD4, or CD8 or in some experiments with CD27, CD127, and CD62L, fixed in 2% paraformaldehyde, and permeabilized with 0.5% saponin before the addition of anti-IFN-γ, anti-TNF-α, or control rat IgG1 (e-Biosciences). Samples were processed with a C6 flow cytometer instrument as described above.

***In vitro* T cell priming assays.** $CD4^+$ T cells and DCs were purified from splenocytes of naive OT-II transgenic mice and WNV-infected mice, respectively, by using anti-CD11c and anti-CD4 magnetic beads according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). $CD4^+$ T cells (2×10^6 cells) were labeled with 0.5 μmol of CFSE (carboxyfluorescein succinimidyl ester; Invitrogen)/liter and mixed with DCs at a 10:1 ratio, with or without OVA peptide (amino acids 323 to 339 [OVA 323-329], 1 μg/ml; GenScript, Piscataway, NJ) for 5 days. The cells were harvested, and $CD4^+$ T cells were analyzed for proliferation based on CSFE dilution. Supernatant was collected at day 3 for analysis of cytokine production.

Adoptive transfer of $CD4^+$ T cells. Single-cell suspensions of $CD4^+$ T cells were prepared from spleens of WNV-infected WT or *Mavs*$^{-/-}$ mice by negative selection using magnetic beads (Miltenyi Biotec). A total of 5×10^6 cells were injected intravenously into naive 6-week-old *Mavs*$^{-/-}$ mice 24 h before infection with 500 PFU of WNV NS4B-P38G. After challenge, the infected mice were monitored twice daily as described above.

Statistical analysis. Survival curve comparisons were performed using Prism software (GraphPad Software, San Diego, CA) statistical analysis, which uses the log-rank test (equivalent to the Mantel-Haenszel test). Values for viral burden, cytokine production, antibody titer, and T cell number experiments were presented as means $\pm$ the standard errors of the mean (SEM). *P* values of these experiments were calculated with a nonpaired Student *t* test or Mann-Whitney test. Statistical significance was accepted at $P < 0.05$.

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